A positive feedback regulatory loop, SA-*AtNAP*-*SAG202*/*SARD1*-*ICS1*-SA, regulates salicylic acid biosynthesis and leaf senescence in Arabidopsis

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ABSTRACT

Salicylic acid (SA) is an important plant hormone that plays critical roles in plant defense and leaf senescence. *SARD1*, a transcription factor (TF), directly regulates *isochorismate synthase 1* (*ICS1*) and SA biosynthesis in defense response. The upstream TF regulating *SARD1* and the regulatory mechanism of SA biosynthesis during leaf senescence are unknown. Here we report that AtNAP, a senescence-specific NAC family TF, directly regulates a senescence-associated gene named *SAG202* (previously reported as *SARD1*). *SAG202*/*SARD1* and *ICS1* are up-regulated during leaf senescence, and are co-induced with inducible overexpression of *AtNAP*. The induction of these genes leads to high levels of SA and precocious senescence in leaves. Individual knockout mutants of *sag202*/*sard1* and *ics1* have markedly reduced SA levels and display a significantly delayed leaf senescence phenotype. Furthermore, SA positively feedback regulates *AtNAP* and *SAG202*/*SARD1*. This research reveals a unique regulatory loop of SA-*AtNAP*-*SAG202*/*SARD1*-*ICS1*-SA in SA biosynthesis during leaf senescence in Arabidopsis. It also provides further evidence for a critical role of SA in controlling leaf longevity.

BIOGRAPHICAL SKETCH

Yaxin Wang was born to Hao Wang and Juping Li in Shijiazhuang, Hebei Province, China, in January 1990. She was raised by her parents in the city, while she spent many summer and winter vacations with her grandparents in a small village in her childhood, which aroused her interest in nature and plants. Then she moved to Tianjin with her parents and attended Nankai University in 2008 where she obtained her Bachelor's Degree in Biotechnology in 2012. Because of her interest in plant biology, she joined Dr. Ningning Wang's lab in College of Life Sciences at Nankai University in her sophomore year and assisted a graduate student's research project on the mechanisms of leaf senescence. This research experience inspired her to go to graduate school in the field of plant biology. Luckily, she joined Dr. Susheng Gan's Lab in Horticulture Department at Cornell University and started her graduate study in August 2012. Her basic knowledge and research skills have been enriched a lot by working with Dr. Gan and she is going further to a PhD program in Plant Biology at UC Davis in the coming September 2014.

Dedicated to my parents,

Juping Li and Hao Wang

ACKNOWLEDGMENTS

To begin with, I would like to thank my advisor, Dr. Susheng Gan, for giving me the opportunity to come to Cornell and join his lab. Dr. Gan has been a very good mentor and taught me a lot about research. He gave me lots of precious advice on developing a project, carrying out experiments, summarizing the results and now writing it into an interesting story. Many thanks also go to my committee members, Dr. Chris Watkins and Dr. Jim Giovannoni for their advice for my research project and unreserved suggestions and help for my PhD application. I also thank Dr. Rayko Halitschke from Department of Ecology and Evolutionary Biology for providing SA extraction protocol and helping with data analysis.

What's more, I'm very thankful to all the lab member's from Dr. Gan's Lab that I worked with during these two years, Youzhen, Xiaoming, Xinyu, Dr. Qin, and also those who have already left, Weifeng Wang, Dr. Caiping Zhao, Dr. Rugang Chen, Dr. Xingwang Liu and Dr. Chunfeng Guan. Thanks for making my time in the lab effective and giving me lots of care like a family. I would also like to thank all the new friends I met at Cornell and all the old friends from Nankai University studying in the States now. You really made my life out of lab more enjoyable and gave me confidence and energy to keep on working.

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CHAPTER 1

INTRODUCTION

Salicylic acid (SA, 2-hydroxy benzoic acid) has pivotal roles in the regulation of many aspects of plant growth and physiological processes such as defense responses, thermogenesis, seed germination, flowering and senescence (1, 2). It is generally accepted that there are two SA biosynthesis pathways in plants: the isochorismate (IC) pathway and the phenylalanine ammonia-lyase (PAL) pathway (3, 4). In Arabidopsis, the IC pathway contributes to most of the SA production induced by pathogens and UV light (5, 6). Although two genes, namely *isochorismate synthase 1* (*ICS1*) and *ICS2,* are involved in isochorismate synthesis in the IC pathway, *ICS1* accounts for approximately 90% of the total amount of isochorismate produced in response to pathogens or UV light (5, 7, 8). It is known that SA levels increase with progression of leaf senescence (9, 10); however, whether the IC pathway operates and functions during leaf senescence is not known.

The regulation of SA biosynthesis and the SA signaling in local and systemic acquired resistance (LAR and SAR) responses against pathogens have been intensively investigated (11–13). Ethylene insensitive 3 (EIN3) and EIN3-like 1 (EIL1) suppress *ICS1* to negatively regulate SA biosynthesis (14), and two closely related transcription factors, calmodulin binding protein 60g (CBP60g) and systemic acquired resistance deficient 1 (SARD1), bind to the core sequence 5'-GAAATTT-3' in the promoter of *ICS1* to positively modulate SAR-related SA production. SARD1 and CBP60g are functionally partially redundant (15, 16). The upstream factor(s) that regulates the expression of *SARD1* and *CBP60g* have yet to be identified.

Leaf senescence is a genetically programmed cell suicide process that is accompanied by mobilization of nutrients released during cell attrition to active growing regions, seeds or trunks (17). The regulation of senescence is rather complex, and it involves activation of thousands of senescence-associated genes (*SAG*s) and/or inactivation of many senescence-down-regulated genes (18, 19). TFs have been shown to have critical roles in regulating *SAG* expression and leaf senescence. For example, *AtNAP*, a NAC family TF gene, acts as a master regulator of leaf senescence because *atnap* null mutants display a 10-day delay in leaf senescence whereas its inducible expression in young leaves readily causes precocious senescence (20). *AtNAP* also has a major role in fruit senescence (21). The direct target genes of *AtNAP* are of significant interest for understanding the molecular circuitry of leaf senescence regulation. Here we report that a senescence up-regulated gene named *SAG202* (At1G73805) is a direct target gene of *AtNAP*; sequence analysis reveals that *SAG202* is identical to *SARD1*. AtNAP physically binds to the promoter region of *SAG202*/*SARD1*, but does not bind to *CBP60g*, and *SAG202*/*SARD1* binds to the promoter region of *ICS1* (but not *ICS2*), as revealed by yeast one-hybrid experiments. Knockouts of *SAG202*/*SARD1* and *ICS1* have lower levels of SA and display a significant delay in leaf senescence whereas inducible overexpression of *SAG202*/*SARD1* leads to high levels of SA and premature leaf senescence. Quantitative PCR analyses further reveal that elevated SA levels can feedback upregulate *AtNAP* and *SAG202*/*SARD1*. These findings suggest that there is a unique feedback regulatory loop consisting of SA-*AtNAP*-*SAG202*/*SARD1*-*ICS1*-SA that modulates the SA biosynthesis to control leaf senescence in Arabidopsis.

CHAPTER 2

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana ecotype Columbia was used in this study. The *atnap* knockout mutants and the related *AtNAP*-inducible expression lines are all in the Columbia background (20). Two T-DNA insertion lines (SALK_052422 and SALK_128476C, Columbia background) were obtained from the Arabidopsis Biological Resource Center (ABRC) at the Ohio State University, USA. As suggested by the Salk Institute Genomic Analysis Laboratory (http://signal.salk.edu/tdnaprimers.2.html), a PCR-based method was used to identify homozygous T-DNA insertion mutants. The T-DNA left border primer G2325 (LBb1.3) and the gene-specific primers, G3832 and G3833 for *sag202-1* (SALK_052422) and G3809 and G3810 for *sag202-2* (SALK_128476C), were used. Plants homozygous for the T-DNA insertion were used in this study. All primers used in this research are listed in Appendix.

Seeds were sterilized with three rinses in 70% ethanol containing 0.01% Triton X-100, and then sown on Petri dishes containing Murashige and Skoog salts with 0.7% w/v phytoagar (Sigma, USA) and appropriate antibiotics. The sown seeds were imbibed at 4 °C for 2 days and then moved to a growth chamber at 22 °C with 60% relative humidity under continuous light (~110 μmol m^{-2} s^{-1}) from a mixture of fluorescent and incandescent bulbs. Approximately 5 days after germination (DAG), seedlings were transplanted to Cornell mix soils (3:2:1 peat moss:

vermiculite: perlite, v/v/v) and grew in a growth chamber. The mutants, transgenic plants, and WT were grown side by side.

Plasmid Construction

For the P_{SAG202}-*GUS* construct, a 2,201-bp promoter fragment of *SAG202* (At1G73805) was amplified from Arabidopsis genomic DNA by PCR with primers G3830 and G3831, cloned into pGEM-T easy vector (Promega, Madison, USA), digested with *Pst I* and *Nco I* and cloned into pBI211 to form pGL8002 (P_{SAG202}-*GUS*).

To generate DEX-inducible *SAG202* overexpression construct, the 1,357-bp full length cDNA of *SAG202* was amplified from Arabidopsis cDNA by RT-PCR with primers G3828 and G3829, ligated to pGEM-T easy vector, sequenced, digested with *Hind III* (Klenow fill-in) and *Pst I*, and cloned into the inducible binary vector pGL1152 (20) that was digested with *Spe I* (Klenow fill-in) and *Pst I* to form pGL8004.

Yeast-one-hybrid assay-related constructs: pGL3175 (for producing GAD-AtNAP fusion protein in yeast) was constructed as described previously (22). To construct pGL8040 (for producing GAD-SAG202 fusion protein in yeast), the *SAG202* coding sequence was amplified from Arabidopsis cDNA by RT-PCR with primers G4020 and G3992, ligated to pGEM-T easy vector, digested with *HindIII* and *XhoI*, and subcloned into the pJG4-5 (23) to form pGL8040. To construct P_{SAG202}-*LacZ*, P_{ICS1}-*LacZ* reporter genes, the 1,122-bp *SAG202* promoter (P_{SAG202}) region and the 1,625-bp *ICS1* promoter region (P_{ICS1}) were amplified from the Arabidopsis

genomic DNA. The pairs of primers used were G3967 and G3918 for P_{SAG202}, and G3993 and G3994 for P_{ICS1}. The amplified fragment was ligated to the pGEM-T easy vector, released from the plasmid with *EcoR I-Sal I* and *EcoR I-Xho I*, respectively, and subcloned into pLacZi-2μ (23) that was digested with *EcoRI-XhoI* to form pGL8017 and pGL8036, respectively. Other *LacZ* reporter gene plasmids containing various truncated *SAG202*, *ICS1*, *CBP60g* and *ICS2* promoters were similarly constructed using the primers listed in Appendix.

Histochemical GUS Staining, Chlorophyll Assay, F_v/F_m Assay and Transcript Analysis

Histochemical GUS staining, Chlorophyll Assay, Fv/Fm Assay were performed as previously described (24, 25).

Total RNA extractions from Arabidopsis leaves and real-time PCR analyses were performed according to (25). Primers used for quantitative RT-PCR were listed in Appendix. Three repetitions were performed for each combination of cDNA samples and primer pairs.

Plant Transformation

Various constructs in binary vectors were transferred into *Agrobacterium tumefaciens* strain ABI1 that were subsequently used to transform Col-0 via the floral-dip method (26). Approximately 30 antibiotics-resistant T1 transgenic lines for each transgene were selected; phenotypic analyses were performed in T2 or advanced generations. Homozygous plants were used in all experiments.

SA Treatment

Col-0 plants, *atnap* and *sag202* mutant plants (all 20 days old) were sprayed with 0.005% Silwet L-77 with or without (control) 5 mM SA. The 5^{th}, 6^{th} and 7^{th} rosette leaves of each plant (counted from bottom) were collected for RNA extraction at different time points after the spray.

Chemical Induction of Gene Expression

Glucocorticoid treatments were performed as previously described (20). Twenty-day-old plants were sprayed with 30μM dexamethasone (DEX, a synthetic glucocorticoid) containing 0.005% Silwet L-77.

Yeast One-Hybrid Assay

Yeast one-hybrid assays were performed as previously described (22). pGL3175 (the *GAD-AtNAP* fusion gene) was co-transformed with different *LacZ* reporter constructs containing different lengths of the *SAG202* promoter fragments into the yeast strain EGY48. Similarly, pGL8040 (the *GAD-SAG202* fusion gene) was co-transformed with different *LacZ* reporter constructs containing different lengths of the *ICS1* promoter fragments into the yeast stain EGY48. The transformants were grown on proper dropout plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) for the blue color development.

SA Quantification

Non-senescing and senescing leaves (0.1-0.3 g) of WT, *atnap*, *sag202*, *ics1*, and the leaves (also 0.1-0.3 g) of *AtNAP* inducible lines (*AtNAP*^{in}), *SAG202* inducible lines (*SAG202*^{in}) and pGL7001 lines (Control) at different time point after chemical induction were collected for analysis of free SA using a protocol described previously (27). 10 µl extracts were injected for analysis using an LC–MS/MS (Quantum Access; Thermo Scientific).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL databases under the following accession numbers: AT1G73805 (*SAG202*, *SARD1*), AT4G10500 (*S3H*), AT1G69490 (*AtNAP*), AT1G74710 (*ICS1*), AT1G18870 (*ICS2*), AT5G26920 (*CBP60g*) and AT3G18780 (*Actin2*, *ACT2*).

CHAPTER 3

RESULTS

***SAG202*/*SAGD1* is up-regulated during leaf senescence.** *SAG202* (*At1G73805*) was initially identified during our analysis of Arabidopsis leaf senescence transcriptome (19) and was later reported as *SARD1* (15, 16). The transcript levels of *SAG202* were examined in leaves that were fully expanded but non-senescing (NS), at early senescence stage (ES, ~5% leaf yellowing), at mid-senescence stage (MS, ~50% yellowing) or at late senescence stage (LS, > 75% yellowing), respectively, using real-time quantitative PCR (Fig. 1A). To further investigate the expression pattern of *SAG202*/*SAGD1*, the *GUS* reporter gene was fused to the 3' end of a 2.2 kb region of the *SAG202* promoter. The GUS staining patterns of the rosette leaves from P_{SAG202}-*GUS* transgenic Arabidopsis showed that *SAG202* was expressed in senescing leaves (Fig. 1B).

Leaf senescence is significantly delayed in *sag202*/*sard1* knockout mutants and precociously accelerated in inducible overexpression lines. Two T-DNA lines, namely *sag202-1* (SALK_052422) and *sag202-2* (SALK_138476C) (Fig. 1C) in which *SAG202* was knocked out (Fig. 1D), were used to investigate the role of *SAG202* in leaf senescence. Compared with the wild type plants, both knockout lines displayed a significant delay in leaf senescence phenotypically (Fig. 1E and F) and physiologically (Fig. 1G and H). Because both knockout lines had the same phenotype, only *sag202-1* was used in the following experiments and referred to as *sag202* for simplicity.

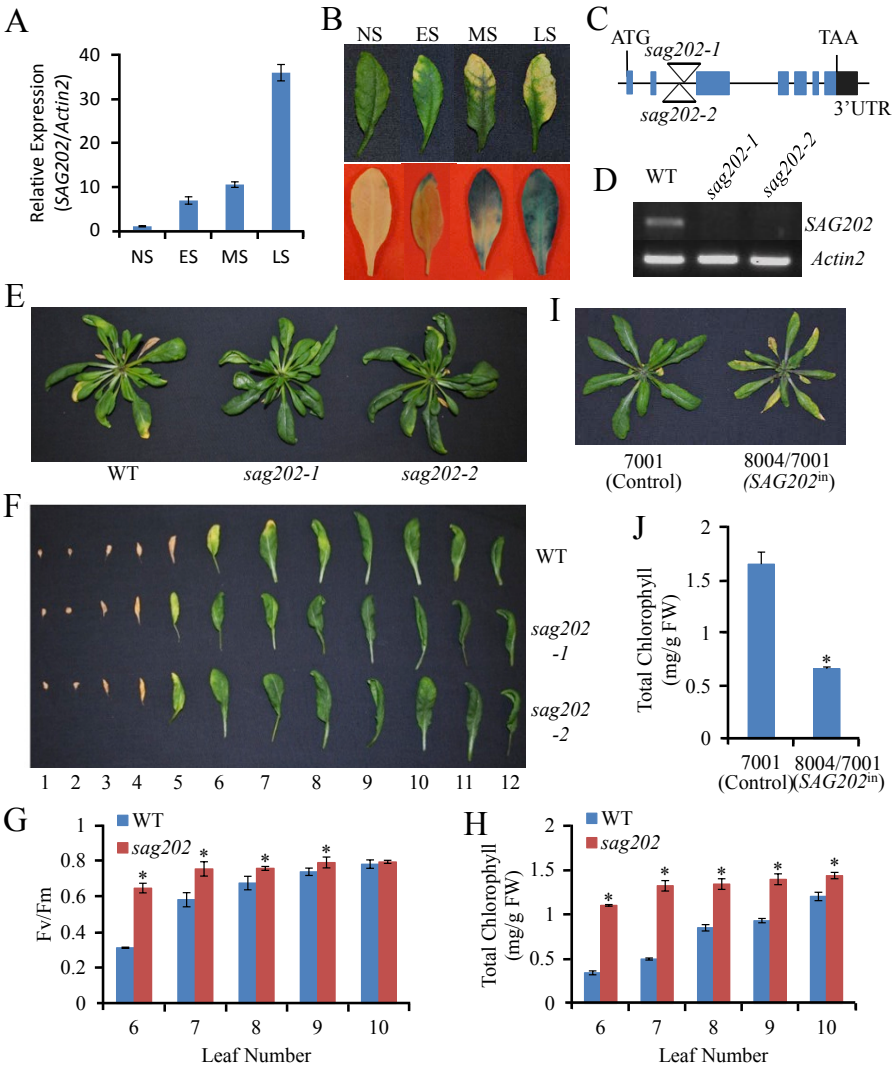
A
Relative Expression (SAG202/Actin2)
40
30
20
10
0
NS
ES
MS
LS
B
NS
ES
MS
LS
C
ATG
sag202-1
TAA
sag202-2
3'UTR
D
WT
sag202-1
sag202-2
SAG202
Actin2
E
WT
sag202-1
sag202-2
I
7001 (Control)
8004/7001 (SAG202in)
F
WT
sag202 -1
sag202 -2
1 2 3 4 5 6 7 8 9 10 11 12
J
Total Chlorophyll (mg/g FW)
2
1.5
1
0.5
0
7001 (Control)
8004/7001 (SAG202in)
G
WT
sag202
Fv/Fm
1
0.8
0.6
0.4
0.2
0
6 7 8 9 10
Leaf Number
H
WT
sag202
Total Chlorophyll (mg/g FW)
2
1.5
1
0.5
0
6 7 8 9 10
Leaf Number

Fig. 1. Molecular and phenotypic analyses of SAG202 in Arabidopsis. (A) qPCR analysis of the transcript levels of *SAG202*/*SARD1* in WT leaves at different developmental stages. NS, fully expanded non-senescing stage; ES, early senescence stage (<25% yellowing); MS, mid-senescence stage (~50% yellowing); LS, late senescence stage (>75% yellowing). Relative expression levels were calculated and normalized with respect to *Actin 2* (*ACT2*) transcripts. Error bars indicate SD of three biological repeats. (B) GUS staining of the fifth leaves from P_{SAG202}-*GUS* transgenic plants at different senescing stages. (C) Diagram of the T-DNA insertion locations of two *sag202*/*sard1* mutants. (D) The expression of *SAG202*/*SARD1* is knocked out in the mutants shown in C as revealed by RT-PCR. (E) Age-matched 35 DAG WT and *sag202*/*sard1* null mutants. DAG, days after germination. (F) Leaves detached from the age-matched 35 DAG plants in E (counted from bottom with the oldest leaf as 1 and the youngest leaf as 12). (G, H) Chlorophyll contents and F_v/F_m of the sixth to tenth rosette leaves of the age-matched 35 DAG plants from WT and the *sag202*/*sard1* mutants. (I) Precocious leaf senescence in *SAG202*/*SARD1*-inducible expression line (8004/7001) (photo was taken 6 days after DEX induction). (J) The chlorophyll contents of the fifth and sixth leaves from WT and *SAG202*/*SARD1* inducible expression lines. Error bars indicate SD of three biological repeats. **P*<0.05 using Student's t-test.

The role of *SAG202* in leaf senescence was also investigated in dexamethasone (DEX) inducible gain-of-function lines. pTA7001 (Control) provides constitutive expression of recombinant GAL4-VP16-GR transcription factor in transgenic plants (20). GAL4 is a domain binding to the GAL4 UAS sequence of a recombinant promoter that directs the expression of the gene of interest (*SAG202* in pGL8004 in this study). VP16 is a transcription activation domain that is masked by glucocorticoid receptor binding domain GR. When DEX (a synthetic glucocorticoid) binds to GR to cause conformational changes, VP16 is able to activate transcription of *SAG202* in plants harboring both pTA7001 and pGL8004. As shown in Fig. 1I and J, treatment of 20-day-old non-senescing plants with 30μM DEX caused precocious leaf senescence in the *SAG202* inducible lines (8004/7001, $SAG202^{in}$) but not in the control lines (7001, Control). qPCR analyses showed that *SAG202* was strongly induced in the *SAG202* inducible lines but not in the control lines (Fig. 2F).

***SAG202*/*SARD1* (but not *CBP60g*) and *ICS1* (but not *ICS2*) are co-induced with *AtNAP*.** *AtNAP* is a NAC family TF that is up-regulated during senescence, and its DEX-inducible expression lines are readily available (20). Upon DEX treatment, the expression of *AtNAP* was significantly induced in *AtNAP* inducible lines but not in control plants (Fig. 2A). qPCR analyses revealed that *SAG202*/*SARD1* and *ICS1* were also induced (Fig. 2B, D) but not *CBP60g*, a gene closely related to *SAG202*/*SARD1*, and *ICS2* were not induced (Fig. 2C, E). *ICS1* was shown to be a target of *SAG202*/*SARD1*(16, 28).

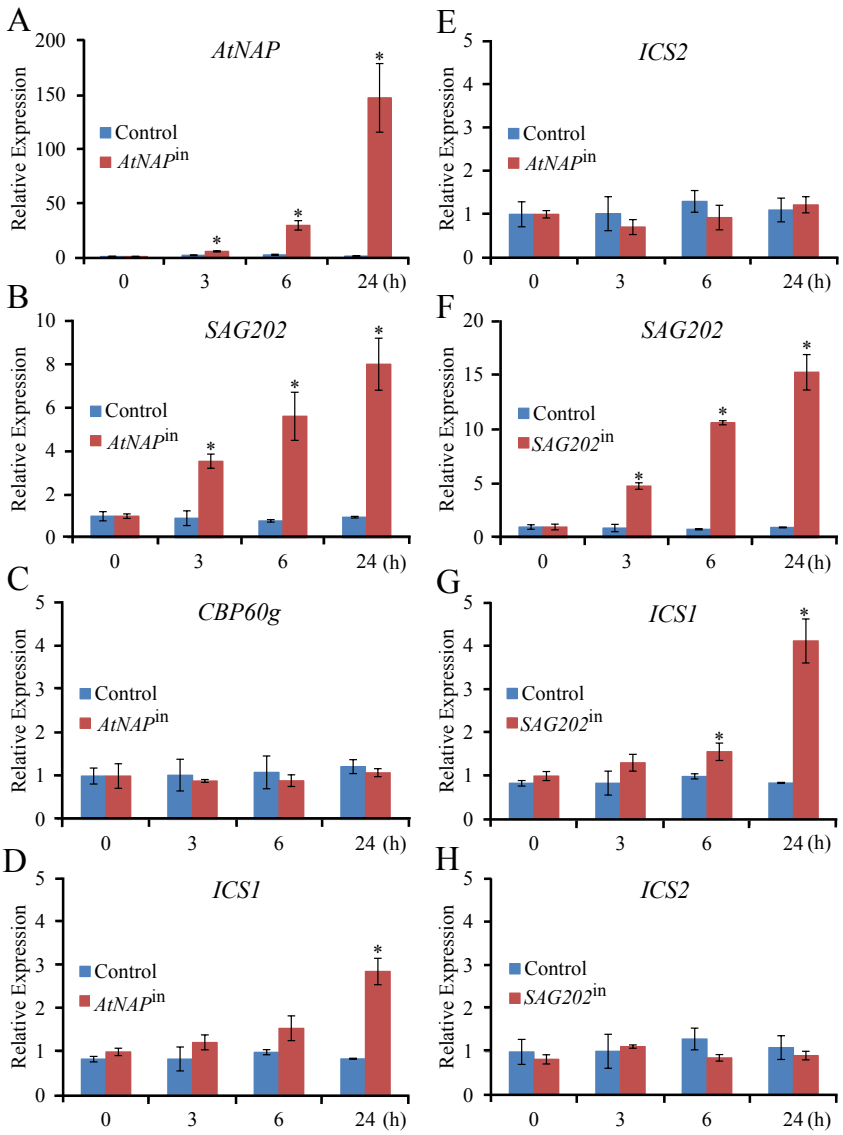

Fig. 2. qPCR analyses of gene expression upon chemical induction of *AtNAP* or *SAG202*/*SARD1*. (A-E) The transcript levels of *AtNAP*, *SAG202*/*SARD1*, *CBP60g*, *ICS1* and *ICS2* in the *AtNAP*^{in} (1167/7001) and control lines (7001) at 0h, 3h, 6h and 24h after DEX treatment. (F-H) The transcript levels of *SAG202*, *ICS1* and *ICS2* in the *SAG202*^{in} (8004/7001) and control lines (7001) at 0h, 3h, 6h and 24h after DEX treatment. Relative expression levels were calculated and normalized with respect to *Actin 2* (*ACT2*) transcripts. Error bars indicate SD of three biological repeats. *P<0.05 using Student's t-test.

AtNAP TF physically binds to the promoter region of *SAG202*/*SARD1* (but not *CBP60g*) *in vivo*. The above co-induction of *SAG202*/*SARD1* with *AtNAP* raised the possibility of *SAG202*/*SARD1* being a direct target gene of *AtNAP*. To test this, we performed yeast one-hybrid experiments in which a series of truncated promoter fragments of *SAG202* (Fig. 3A) were cloned in front of a *LacZ* reporter gene as promoter baits to form various reporter constructs; the *AtNAP* coding sequence was fused with the yeast GAL4 activation domain (GAD) to form the effector GAD-AtNAP construct (22). The AtNAP TF was able to physically bind to a specific region of the *SAG202* promoter that is very similar to the 9-bp *AtNAP* core binding sequence, 5'CACGTAAGT3', in the promoter of *SAG113* (22) (Fig. 3A). In contrast, AtNAP TF did not bind to the promoter of *CBP60g* (Fig. 3A).

SAG202/SARD1 TF binds to the promoter of *ICS1* but not *ICS2 in vivo*. SAG202/SARD1 TF has been previously shown to directly regulate *ICS1* using chromatin immunoprecipitation (ChIP) and promoter sequence analysis (16, 28). Here we used yeast one-hybrid system to show that the SAG202/SARD1 TF was able to bind to a 103-bp region of the promoter of *ICS1* but not *ICS2* (Fig. 3B).

Both *AtNAP* and *SAG202*/*SARD1* are positively regulated by SA. The above data revealed a regulatory chain consisting of *AtNAP*-*SAG202*/*SARD1*-*ICS1* operating to produce SA during leaf senescence. If so, knocking out of an up-stream gene should effect the expression of its down-stream genes. We thus performed qPCR to analyze the expression levels of these genes in wild type, *atnap* null mutants, *sag202*/*sard1* null mutants and *ics1* null mutants at different senescence stages (Fig. 4). As expected, the transcript levels of both *SAG202*/*SARD1* and *ICS1*

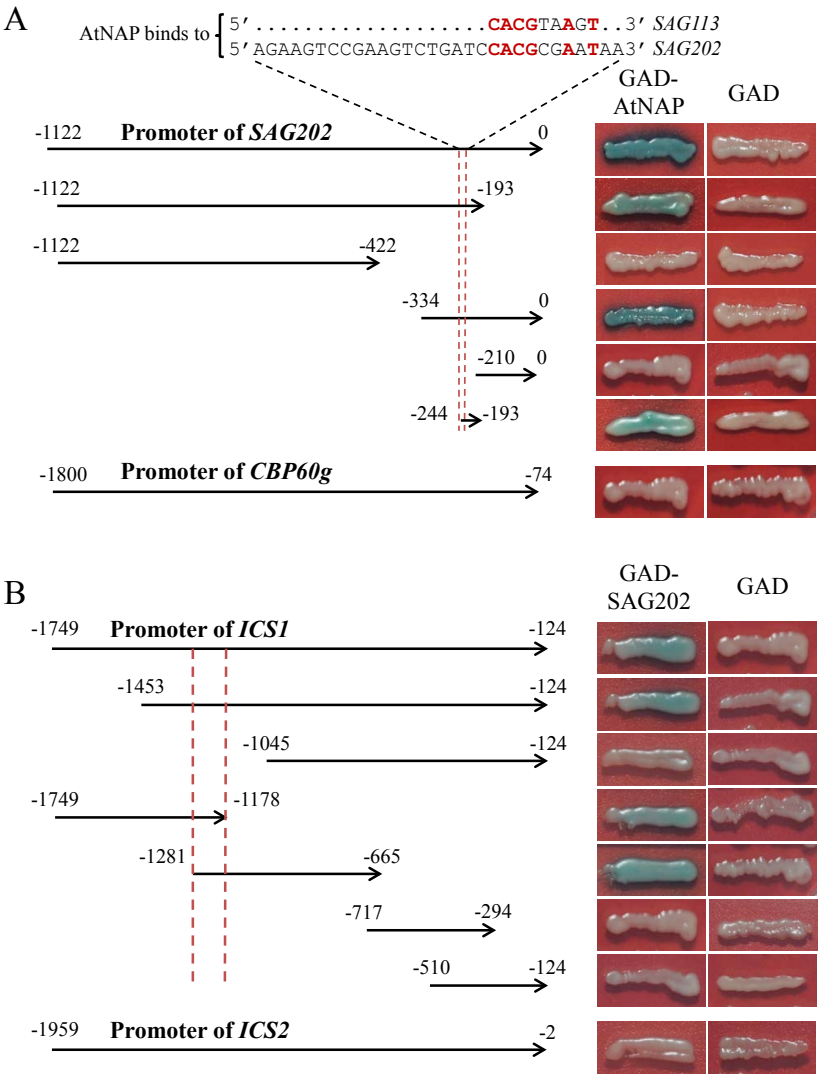
A
AtNAP binds to
5′ . . . . . CACGTAAGT . . 3′ SAG113
5′ AGAAGTCCGAAGTCTGATCCACGCGAATAA 3′ SAG202
GAD-AtNAP
GAD
-1122 Promoter of SAG202 0
-1122 -193
-1122 -422
-334 0
-210 0
-244 -193
-1800 Promoter of CBP60g -74
B
GAD-SAG202
GAD
-1749 Promoter of ICS1 -124
-1453 -124
-1045 -124
-1749 -1178
-1281 -665
-717 -294
-510 -124
-1959 Promoter of ICS2 -2

Fig. 3. Yeast one-hybrid analyses of bindings of AtNAP and SAG202/SARD1 to promoter truncations of their respective target genes. (A) Binding of AtNAP to the *SAG202* promoter truncations. The *LacZ* reporter gene driven by various *SAG202* promoter truncations was used to test the binding ability of the GAD-AtNAP fusion protein. Red dash lines indicate promoter sequence that is highly conserved to the 9-bp AtNAP binding site of the *SAG113* promoter (22). The translation start site was numbered as +1. The *CBP60g* promoter (1,727-bp in length) was also tested. (B) Binding of *SAG202*/*SARD1* to the *ICS1* promoter truncations. The *LacZ* reporter gene driven by various *ICS1* promoter truncations was used to test binding ability of the GAD-SAG202 fusion protein. Red dash lines indicate the 103-bp promoter region containing the *SAG202*/*SARD1* binding sequence. The *ICS2* promoter (1,958-bp in length) was also tested.

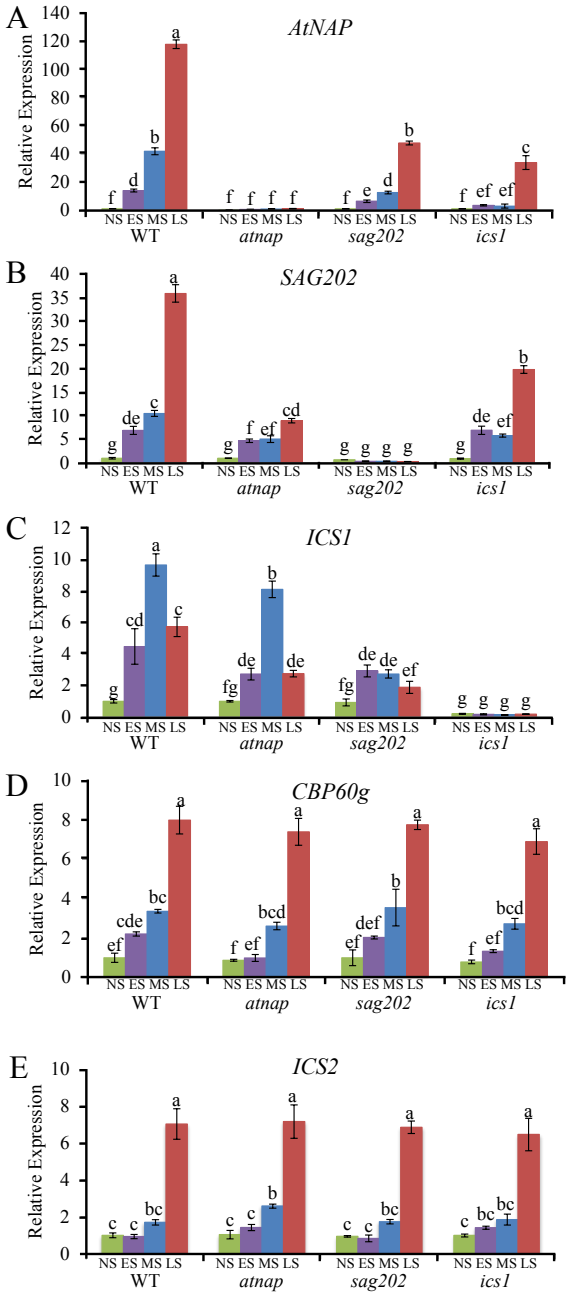

Fig. 4. qPCR analyses of transcript levels of *AtNAP*, *SAG202*/*SARD1*, *ICS1*, *CBP60g and ICS2* in different mutants during senescence. Relative expression levels were calculated and normalized with respect to *Actin 2* (*ACT2*) transcripts. Error bars indicate SD of three biological repeats. Significant (P<0.05) differences between means are indicated by different letters using Tukey's HSD test.

were significantly reduced in the absence of *AtNAP* (Fig.4B and C); similarly, the *ICS1* expression levels were remarkably lowered in the *sag202*/*sard1* knockout mutants (Fig. 4C). In contrast, the expression levels of *CBP60g* and *ICS2*, two genes outside of the regulatory chain, were not altered in any of the mutant backgrounds (Fig. 4D and E).

Interestingly, the expression levels of *AtNAP* in either *sag202*/*sard1* or *ics1* null mutants were reduced (Fig. 4A), and the transcript levels of *SAG202*/*SARD1* in leaves at the mid-senescence stage (MS) in the *ics1* background were also decreased (Fig. 4C). These data suggested the possibility that the end product SA of the regulatory chain might feedback regulate those genes. To test this hypothesis, we analyzed the expression levels of *AtNAP*, *SAG202*/*SARD1*, *ICS1* in WT, *atnap* mutants and *sag202*/*sard1* mutants upon SA treatments. As shown in Fig. 5, *AtNAP* and *SAG202*/*SARD1* were significantly induced by SA while the induction of *ICS1* in the *sag202*/*sard1* null mutants was not so significant, suggesting that *AtNAP* and *SAG202*/*SARD1* were positively feedback regulated by SA.

Free SA levels were reduced in *atnap* and *sag202*/*sard1* mutants and elevated in *AtNAP*^{in} and *SAG202*^{in} inducible lines. The free SA levels in fully expanded non-senescing leaves (NS) and senescing leaves (S) of WT, *atnap*, *sag202*/*sard1* and *ics1* mutants were quantitatively analyzed using LC-MS/MS. The SA levels in the senescing leaves were significantly reduced in these null mutants but remained unchanged in the non-senescing leaves of any of the plants (Fig. 6A).

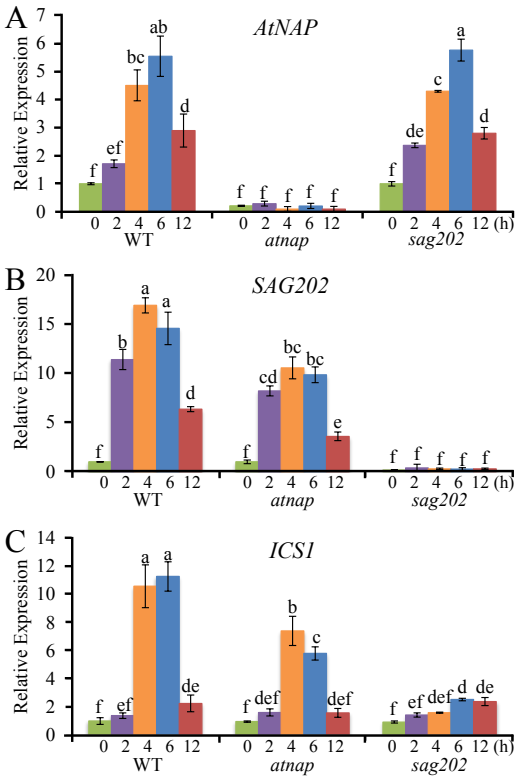

Fig. 5. Induction of *AtNAP*, *SAG202*/*SARD1* and *ICS1* by exogenous SA. (A-C) qPCR analyses of transcript levels of *AtNAP*, *SAG202*, *ICS1* induced by SA treatment in fully expanded non-senescing leaves of WT, *atnap* and *sag202* null mutants. Relative expression levels were calculated and normalized with respect to *Actin 2* (*ACT2*) transcripts. Error bars indicate SD of three biological repeats. Significant (P<0.05) differences between means are indicated by different letters using Tukey's HSD test.

The free SA levels in leaves with inducible expression of *AtNAP* (1167/7001) or *SAG202*/*SARD1* (8004/7001) were also quantitated. As shown in Fig. 6B, the SA levels were significantly increased readily one day after the inducible expression of either *AtNAP* or *SAG202*/*SARD1*.

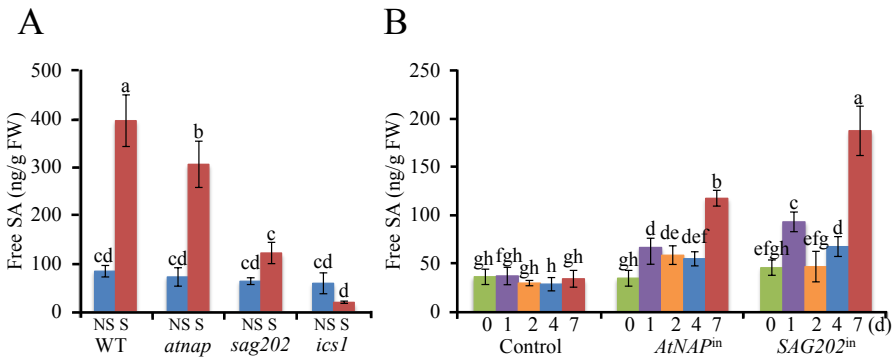

Fig. 6. LC-MS/MS analyses of free SA levels in WT, *atnap*, *sag202*/*sard1*, *ics1* null mutants, and in *AtNAP*^{in} and *SAG202*^{in} lines. (A) Free SA levels in non-senescing (NS) and senescing rosette leaves (S, ~50% yellowing) of WT, *atnap*, *sag202* and *ics1* mutants, respectively. (B) Free SA levels in young leaves of *AtNAP* inducible lines (*AtNAP*^{in}) and *SAG202* inducible lines (*SAG202*^{in}) at 0, 1, 2, 4, and 7 days after DEX induction. Error bars indicate SD of three biological repeats. Significant (P<0.05) differences between means are indicated by different letters using Tukey's HSD test.

CHAPTER 4

DISCUSSION

Our research reported here unravels a unique positive feedback regulatory chain, SA-*AtNAP*-*SAG202*/*SARD1*-*ICS1*-SA, that operates during leaf senescence (and possibly during defense responses against pathogens). It also provides strong lines of evidence for an important role of SA in leaf senescence.

***AtNAP* is a direct regulator immediately upstream of *SAG202*/*SARD1*.** Because of the significant role of SA in plant defense, much research has been performed to decipher its biosynthesis and signaling in plant (11, 12, 29). There are two pathways leading to the production of SA in plants: one from phenylalanine and the other from chorismate via isochorismate (IC) (6). In Arabidopsis, the IC pathway contributes predominantly to SA accumulation during defense responses and *isochorismate synthase 1* (*ICS1*) has the major role in this accumulation (8). Further studies showed that SARD1 and CBP60g bind to the promoter of *ICS1* to regulate this gene's expression (15, 16). Most recent studies revealed that SARD1 binds to a specific 6-bp sequence, 5'GAAATT3', of the *ICS1* promoter (28). Which TFs regulate *SARD1* is unknown. Our research addressed this question by identifying a NAC family TF named AtNAP that is a direct upstream regulator of *SARD1*; this was supported by at least two lines of evidence: (i) the yeast one-hybrid experiments showed that AtNAP could physically bind to a promoter region of *SAG202*/*SARD1* that contains a highly conserved sequence to which AtNAP binds (Fig. 3A), and (ii) *SAG202*/*SARD1* was co-induced when *AtNAP* was chemically induced (Fig. 2B). Interestingly, *CBP60g*, the close homolog of *SAG202*/*SARD1,* is unlikely to

be directly regulated by *AtNAP* because AtNAP could not bind to the promoter region of *CBP60g* (Fig. 3A) and because *CBP60g* was not co-induced with *AtNAP* (Fig. 2C).

In addition to uncovering the *AtNAP-SAG202*/*SARD1* chain, we also provided new lines of evidence that *SAG202*/*SARD1* physically binds to the promoter of *ICS1* (but not *ICS2*) as shown by our yeast one-hybrid experiment results (Fig. 3B), and by induction of the expression of *ICS1* (Fig. 2D, G) but not *ICS2* (Fig. 2E, H) through chemical activation of *AtNAP* or *SAG202*/*SARD1*.

These data reveal a unique regulatory chain consisting of *AtNAP-SAG202*/*SARD1-ICS1*, which significantly advanced our understanding of molecular regulatory mechanism of the SA biosynthesis.

The *AtNAP-SAG202*/*SARD1-ICS1* regulatory chain operates and functions during leaf senescence. It is known that the SA levels are higher in senescing leaves than in non-senescing leaves and SA has an important role in controlling leaf senescence in Arabidopsis (9, 10). *SARD1-ICS1* has been shown to contribute to the SA production during defense responses (8, 15, 16), but whether it, together with its upstream component *AtNAP*, also operates and functions during leaf senescence was not understood. This research provided several lines of evidence for the regulatory chain's operation in leaf senescence. The first line of evidence comes from the qPCR analysis of transcript levels of individual genes in the chain. As shown in Fig. 4, the expression levels of *AtNAP*, *SAG202*/*SARD1* and *ICS1* were all up-regulated with the progression of leaf senescence. The second line of evidence is from the quantification of SA levels in senescing leaves of respective null mutants. When individual genes were knocked out,

the SA levels in senescing leaves were all significantly reduced (Fig. 6A). The closer of the gene to SA biosynthesis in the regulatory chain, the more dramatic reduction of the SA levels (Fig. 6A); this could be due to the possibility in which, for example, *SAG202*/*SARD1* may also be regulated by other factors in addition to *AtNAP*.

SA positively feedback regulates the *AtNAP-SAG202/SARD1-ICS1* chain. In the absence of either *SAG202*/*SARD1* or *ICS1*, the transcript levels of *AtNAP* were significantly reduced in senescing leaves, the late senescence (LS) leaves in particular (Fig. 4A). Similarly, the expression levels of *SAG202*/*SARD1* in the *ics1* null background were also decreased (Fig. 4B). These data suggest that SA, the end product of the regulatory chain, may positively feedback regulate *AtNAP* and *SAG202*/*SARD1* as shown in Fig 7. This feedback regulation is supported by the fact that exogenous SA remarkably elevated the *AtNAP* transcript levels in both WT and the *sag202*/*sard1* null mutants (Fig. 5A). In the absence of *AtNAP*, external SA was able to drastically induce the *SAG202*/*SARD1* expression (Fig. 5B), suggesting that SA has a direct positive feedback regulation on *SAG202*/*SARD1* beyond *AtNAP*. In contrast, in the absence of *SAG202*/*SARD1*, the *ICS1* expression levels were not significantly altered by the external SA (Fig. 5C), indicating that *ICS1* is not positively feedback regulated by SA.

The pivotal role of SA in leaf senescence is reinforced. Previous correlative studies showing higher levels of SA in senescing leaves compared with those in non-senescing leaves, delayed leaf senescence in the NahG or S3H plants in which a SA-degrading enzyme of bacterial or Arabidopsis origin was overexpressed, and the accelerated leaf senescence in the *s3h* null plants

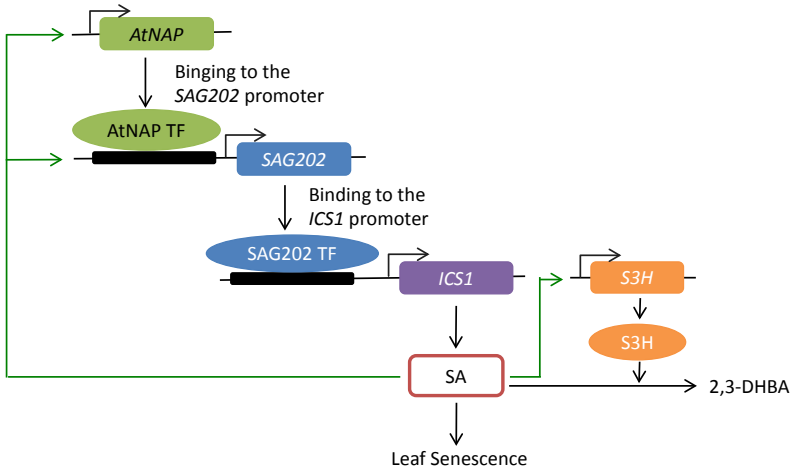

Fig. 7. A working model of SA-*AtNAP-SAG202*/*SARD1-ICS1*-SA-*S3H* positive feedback loop in leaf senescence in Arabidopsis. At the onset of and during leaf senescence, AtNAP TF physically binds to the promoter of *SAG202*/*SARD1* to direct the target gene expression. Subsequently the SAG202/SARD1 TF activates its direct target gene *ICS1* that is involved in the SA biosynthesis. The produced SA in turn feedback upregulates both *AtNAP* and *SAG202*/*SARD1*. When the SA levels increase to a threshold, *S3H* (encoding an SA 3-hydroxylase) is induced (9) to prevent over accumulation of SA. Too high levels of SA will cause hypersensitive response (HR)-like fast cell death. Leaf senescence is a slow programmed cell death process to allow nutrients released from degradation of proteins and other macromolecules to be recycled to active growing region or storage organs (17).

in which SA were over-accumulated have suggested an important role of SA in leaf senescence (9, 10). In this research, we found that when any of the genes in the regulatory loop is knocked out, the endogenous SA levels were significantly reduced (Fig. 6A) and the leaf longevity was drastically prolonged (Fig. 8, 17), whereas, when *AtNAP* and *SAG202/SARD1* were individually chemically induced, the endogenous SA levels were enhanced (Fig. 6B) and the plants displayed precocious leaf senescence (Fig. 1I and J, 17). These data reinforces the SA's role in promoting leaf senescence.

The *SAG202*/*SARD1-ICS1* regulatory chain but not the *AtNAP* component may be shared between leaf senescence and defense response. SA has been identified as a key hormone in response to various biotic and abiotic stresses such as pathogen infection (13, 30), UV-B exposure (7), ozone exposure (31), and leaf senescence (10, 32). It has been generally accepted that leaf senescence and plant defense response may share some components in SA biosynthesis and signaling (9, 10, 33). As discussed above, the newly uncovered regulatory chain in this report operates during leaf senescence, and the *SAG202*/*SARD1-ICS1* node has been clearly shown to function in plant defense response (8, 15, 16). To investigate whether *AtNAP* also has any role in defense, we inoculated leaves of *atnap*, *sag202*/*sard1*, *ics1* mutants and WT with *Pseudomonas syringae* pv. Tomato DC3000 and found that the defense response in the *atnap* mutant was not changed compared with that in WT while the *sag202*/*sard1* and *ics1* mutants became more susceptible to the pathogen infection (Fig. 9). These data strongly suggest that the *SAG202*/*SARD1-ICS1* regulatory chain is shared by leaf senescence and defense response and that the up-stream component *AtNAP* appears to be leaf senescence specific (Fig. 10). Further analyses in this regard are needed.

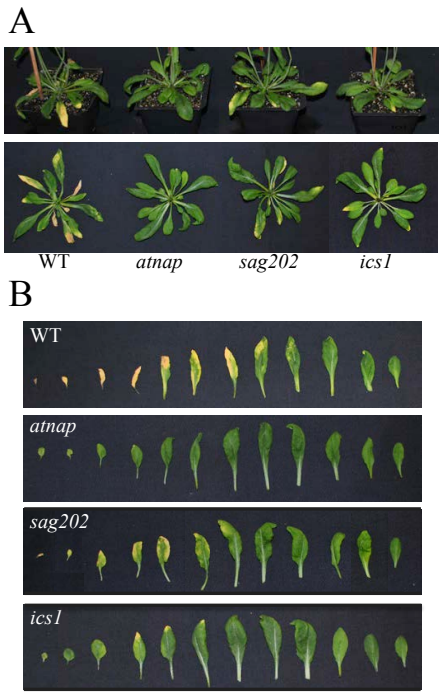

Fig. 8. Delayed leaf senescence phenotype in *atnap*, *sag202*/*sard1* and *ics1* null mutants. (A) Age-matched 40 DAG WT, *atnap*, *sag202* and *ics1* null mutants. (B) Phenotypes of leaves detached from the age-matched 40 DAG plants in A. DAG, days after germination.

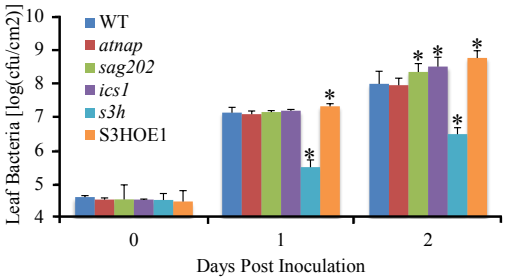

Fig. 9. Growth of *P. syringae* pv. Tomato DC3000 in leaves of WT, *atnap*, *sag202*/*sard1*, *ics1*, *s3h* null mutants and S3HOE1 transgenic plants. The number of colony-forming units (cfu) per square centimeter of leaf area was determined 0, 1, and 2 days after inoculation. Error bars indicate SD of three biological repeats. **P*<0.05 using Student's t-test. The *s3h* null mutant (resistant to the infection) and S3HOE1 transgenic plants (susceptible) (9) were included for comparison purpose.

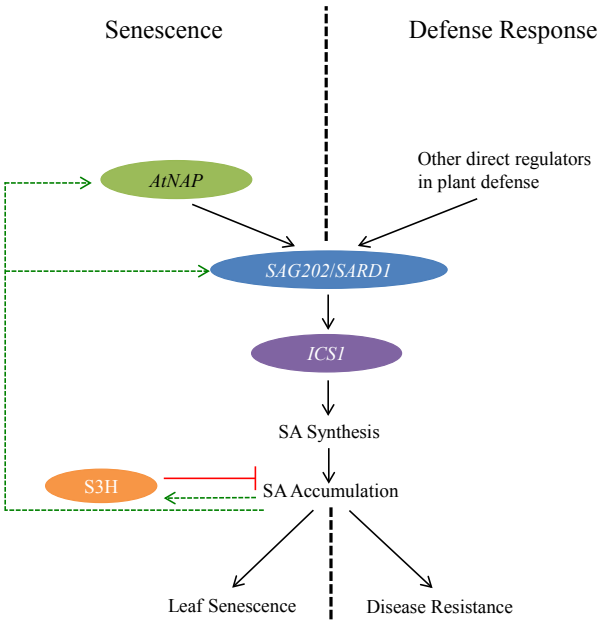

Fig. 10. A diagram showing convergence and divergence between leaf senescence and defense response with regard to the newly uncovered SA-*AtNAP-SAG202/SARD1-ICS1*-SA regulatory loop. SA-*AtNAP-SAG202-ICS1*-SA-*S3H* feedback loop is activated to modulate endogenous SA levels in leaf senescence. In defense responses, SA is also synthesized through *SAG202-ICS1*-SA regulatory pathway.

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APPENDIX

Primers used in this research

Name	Sequence (5'→3')	Engineered Restriction Enzyme Sites	Purpose
G3830	CTGCAGTAGAGAATGTCAGTTATATGATTGGC	the underlined section is an engineered PstI site	P_{SAG202}-*GUS*
G3831	CCATGGGGAATTGTTCTGGTGAGTTGTG	the underlined section is an engineered NcoI site	
G3828	CTGCAGATGGCAGGGAAGAGGTTATTTC	the underlined section is an engineered HindIII site	*SAG202* inducible lines
G3829	GAGCTCTTAGAAAGGGTTTATATGATTTTG	the underlined section is an engineered PstI site	
G4020	AAGCTTACATGGCAGGGAAGAGGTTAT	the underlined section is an engineered HindIII site	*GAD-SAG202*
G3992	CTCGAGCTTCCAATACTAACGTAGATGAGGAT	the underlined section is an engineered XhoI site	
G3967	GAATTCGCACGACAAGTTTTGAGAGGATG	the underlined section is an engineered EcoRI site	P_{SAG202}-*LacZi*
G3918	GTCGACGGAATTGTTCTGGTGAGTTGTG	the underlined section is an engineered SalI site	
G3957	GTCGACATACGATCGATCCGGTCCGT	the underlined section is an engineered SalI site	
G3958	GAATTCGGAAGATCGGAACCGTCCAT	the underlined section is an engineered EcoRI site	
G4083	CTCGAGACAACCTGGCAATATCCAAAG	the underlined section is an engineered XhoI site	
G4108	GAATTCGTCTCCCTATTTATGACGCCAT	the underlined section is an engineered EcoRI site	
G4109	GAATTCGATTATTCGCGTGGATCAGACTTCGG	the underlined section is an engineered EcoRI site	
G3993	GAATTCAAGATTTCGAACTAAAGC	the underlined section is an engineered EcoRI site	P_{ICS1}-*LacZi*
G3994	CTCGAGATAGGGGACTGATGTAGCAG	the underlined section is an engineered XhoI site	
G4028	GAATTCGGATTATCTGCAAGACTTC	the underlined section is an engineered EcoRI site	
G4029	GAATTCGGTGAGCCGTCTTAATC	the underlined section is an engineered EcoRI site	
G4031	GAATTCGAGCCTAAGTGGGTTTCC	the underlined section is an engineered EcoRI site	
G4032	CTCGAGGCAAAGAGTGGAGAGG	the underlined section is an engineered XhoI site	
G4079	GAATTCGTGCAAACCGCTTCCGTATCAAAC	the underlined section is an engineered EcoRI site	
G4080	CTCGAGGGGTGTTGTATGCTAGACGACTCTTC	the underlined section is an engineered XhoI site	
G3149	CGTAAAGCATCAACGAAACG		*AtNAP* qPCR
G3835	TGGAAGTTTCATCGACGTCAT		
G3875	CCTCAACCAGCCCTACGTTA		*SAG202* qPCR
G3876	TAGTGGCTCGCAGCATATTG		
G3877	GTGACATCATGGTTCTCTGTTACC		*ICS1* qPCR
G3878	AGGCCTGCCCTAGTTACAACC		
G3826	AGCAATCGATTGGCTTCAT		*ICS2* qPCR
G3827	TCCATAGGCACGAATCAGAG		
G4061	AGGTCCTTACAGTGCCCGCAAG		*CBP60g* qPCR
G4062	CAGGGTGGACCGTTGAGCTTG		
G3053	AGTGGTCGTACAACCGGTATTGT		*Actin2* qPCR
G3054	GATGGCATGAGGAAGAGAGAAAC		